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1. Your reference NW/7609

2. Patent application number *(The Patent Office will fill in this part)* 0306394.8 20 MAR 2003

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

The University of Nottingham
University Park, Nottingham, NG7 2RD,
United Kingdom.

Patents ADP number *(if you know it)* 7610223001

If the applicant is a corporate body, give the country/state of its incorporation United Kingdom

4. Title of the invention

Carnitine Retention

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

Swindell & Pearson

48 Friar Gate,
Derby DE1 1GY

Patents ADP number *(if you know it)* 00001578001

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day / month / year)*

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a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
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See note (d))

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Patents Form 1/77

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Continuation sheets of this form	0
Description	13
Claim(s)	0
Abstract	0
Drawing(s)	4 +4

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Translations of priority documents	
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	
Request for preliminary examination and search *(Patents Form 9/77)*	
Request for substantive examination *(Patents Form 10/77)*	
Any other documents *(please specify)*	

11. I/We request the grant of a patent on the basis of this application.

Signature Swindell & Pearson Date 19/03/03

Swindell & Pearson

12. Name and daytime telephone number of person to contact in the United Kingdom N. Womsley - 01332 367051

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Carnitine Retention

This invention relates to carnitine retention in biological tissue. More particularly, but not exclusively, the invention relates to compositions and methods of increasing carnitine retention in the animal and/or human body.

It is known that carnitine is essential in muscle metabolism function. In particular the muscle store of carnitine is important for energy production in muscle. If the store of carnitine declines, the function of the muscle can be impaired. Indeed, patients with muscle carnitine deficiency experience premature fatigue and weakness.

Previous studies (Harper *et al*, 1988, Segre *et al* 1988, Rebouche 1991), where oral doses of L-carnitine between 2 and 6 g were administered, demonstrate peak plasma concentrations ~3 h after ingestion and state a bioavailability of less than 20%. This poor absorptive status may be due to the fact that intestinal absorption of L-carnitine is normally near saturation (Taylor, 2001). Further studies (Rebouche *et al*, 1994, Brass *et al*, 1994) showed that if plasma carnitine concentrations exceed maximum renal reabsorption (60-100 µmol/L), the excess is excreted in the urine with a clearance approximating the glomerular filtration rate. From these features of carnitine's pharmacokinetics, and the fact that the normal plasma carnitine concentration of 40-50 µmol/L is sufficient to yield near maximal rates of skeletal muscle carnitine uptake (K_M 6.4 µM in isolated cells, Georges *et al*, 2000), it can be predicted that oral L-carnitine supplementation would have little, if any, impact on skeletal muscle carnitine content or metabolism in humans (Brass, 2000).

A study by Vukovich *et al* (1994) showed that L-carnitine supplementation (6 g every day for up to 2 weeks) resulted in no significant increase in resting skeletal muscle carnitine content and suggested that there was already an adequate amount of carnitine within the muscle to support fatty acid oxidation

during exercise. However, Vukovich's study did not look at carnitine status in muscle. The results seen in a study by van Loon *et al*, 2001 which did look at carnitine status in muscle do suggest, in contradiction to Vukovich, that there is not enough carnitine within the muscle to support fatty acid oxidation during exercise at workloads above 70% maximal oxygen consumption (VO_2 max). Other studies (Grieg *et al*, 1987, Oyono-Enguelle *et al*, 1988, Soop *et al*, 1988, Wyss *et al*, 1990, Decombaz *et al*, 1993), involved orally supplemented 3-5 g L-carnitine, in subjects with varying levels of fitness, over 5-28 days and measured the effects on various endpoints of exercise. Findings from these studies concluded that there was no effect of L-carnitine on VO_2 max, RQ, maximal exercise, fatty acid utilisation, glucose utilisation, lactate, perceived exertion, or heart rate. However, again these studies did not measure skeletal muscle carnitine content. If skeletal muscle carnitine content did not increase then clearly there would not be an affect on skeletal muscle metabolism and thus, an enhancement in the endpoints measured.

In contrast to these findings, Marconi *et al* (1985) did observe a slight but significant increase in VO_2 max in competitive walkers, after oral supplementation of 4 g L-carnitine every day for 2 weeks, which they concluded was most likely due to an increase in TCA flux as lipid metabolism did not change. Vecchiet *et al* (1990) also observed an increase in VO_2 max. However, only a single dose (2 g, orally) was supplemented an hour before exercise and, due to the features of carnitine's pharmacokinetics, it is highly unlikely the observed effects were a result of an increase in skeletal muscle carnitine, which was not measured.

According to one aspect of the present invention there is provided a composition for influencing carnitine retention in biological tissue, the composition comprising a carnitine substance and an agent to increase sodium-potassium ATPase pump activity in the tissue.

The invention further provides a composition for influencing carnitine transport into biological tissue, the composition comprising a carnitine substance

to increase blood/plasma carnitine concentration and an agent to increase the activity of a carnitine transport protein.

According to a further aspect of the present invention there is provided a composition for increasing carnitine retention in the animal and/or human body, the composition comprising a carnitine substance and an agent to increase blood/plasma insulin concentration.

The invention also provides a method of influencing carnitine retention in biological tissue, in particular tissue of the animal and/or human body, the method comprising administering to the tissue a carnitine substance and an agent operable to increase sodium-potassium ATPase pump activity in the tissue.

The invention further provides a method of increasing carnitine retention in the animal and/or human body, the method comprising administering to the body a carnitine substance and an agent to increase blood/plasma insulin concentration.

The invention still further provides a method of influencing carnitine transport into biological tissue, the method comprising administering to the body a carnitine substance to increase blood/plasma carnitine concentration and an agent to increase the activity of a carnitine transport protein.

Preferably the method increases carnitine retention in the tissue by increasing the transportation of the carnitine substance, or a derivative thereof into tissue cells. Preferably transportation is increased by stimulation of a sodium dependent transport protein and substantially simultaneously increasing blood/plasma carnitine concentration.

Preferably the agent is operable to increase sodium dependent carnitine uptake into tissue cells, in particular skeletal muscle, liver and/or kidney cells.

The agent may be operable to increase insulin activity in the tissue, desirably by increasing the amount of insulin in the blood/plasma. The agent may comprise carbohydrate or an active derivative thereof. Alternatively, or in addition, the agent may comprise amino acid and/or protein.

Preferably the method involves oral administration and desirably ingestion of the carnitine substance and agent, desirably but not necessarily simultaneously.

According to a still further aspect of the present invention there is provided a food supplement comprising a carnitine substance and an agent as described in any of the preceding paragraphs.

The invention further provides a composition for use in the manufacture of a medicament for influencing carnitine retention in biological tissue, the composition comprising a carnitine substance and an agent to increase sodium-potassium ATPase pump activity in the tissue.

The invention also provides a composition for use in the manufacture of medicament for influencing carnitine transport into biological tissue, the composition comprising a carnitine substance to increase blood/plasma carnitine concentration and an agent to increase the activity of a carnitine transport protein.

There is also provided a composition for use in the manufacture of a medicament to influence carnitine retention in the animal and/or human body, the composition comprising a carnitine substance and an agent to stimulate insulin release and activity in the body.

The invention also relates to the use of a carnitine substance and an agent as described in any of the preceding paragraphs for influencing carnitine retention in human and/or animal tissue.

Carnitine is also provided for use in a method substantially as described in any of the paragraphs above.

A kit is provided according to this invention comprising a carnitine substance and an agent substantially as described in any of the paragraphs above.

According to another aspect of the present invention, there is provided a carnitine substance for use in administration to the human and/or animal body with an agent as described in any of the paragraphs above.

Desirably, the carnitine substance comprises one or more of carnitine, a functional equivalent of carnitine, an active derivative of carnitine or carnitine analogue. A preferred embodiment may comprise one or more of L-carnitine, a functional equivalent of L-carnitine, an active derivative of L-carnitine or an analogue thereof.

Preferably the agent is a carbohydrate or a derivative of a carbohydrate. The carbohydrate is preferably a simple carbohydrate, which may be a simple sugar. Conveniently, the carbohydrate comprises glucose, but other sugars can be used, for example sucrose or fructose.

Preferably between 10 and 150 times the amount by weight of carbohydrate is administered to one unit of carnitine substance. Preferably approximately 3 g of the carnitine substance is administered with a total of approximately 30 to 450 g of agent, the agent being administered to achieve substantially simultaneous elevation of insulin and carnitine concentrations in the blood/plasma.

The composition may be provided in a solution which may be an aqueous solution.

An embodiment of the invention will now be described by way of example only with reference to the accompanying drawings, in which:-

Fig. 1 shows serum insulin concentrations following carnitine ingestion with Control (○) and CHO (●);

Fig. 2 shows urinary total carnitine (TC), free carnitine (FC) and acylcarnitine (AC) excretion in mg over 24 hours following carnitine ingestion with Control (□) and CHO (■);

Fig. 3 shows plasma TC concentration measured over 7 hours following carnitine ingestion with Control (□) and CHO (■). The arrows A, B, C, D indicate time of ingestion of drink; and

Fig. 4 shows the area under the plasma-time curves (AUC) for total carnitine (TC), free carnitine (FC) and acylcarnitine (AC) measured over 7 hours following carnitine ingestion with Control (□) and CHO (■).

Referring to the figures, the invention provides a composition, methodology and uses of a composition to influence carnitine retention in tissue such as muscle, liver and kidney tissue in the animal and/or human body which comprise a carnitine substance and an agent to increase blood/plasma insulin concentration with a view to increasing sodium-potassium ATPase pump activity in tissue, and thereby sodium dependent carnitine transport.

The carnitine substance comprises one or more of L-carnitine, a functional equivalent of L-carnitine, an active derivative of L-carnitine or an analogue thereof.

The agent can be anything which acts to increase insulin concentration, including amino acids and protein. However in this embodiment the agent is a carbohydrate such as a sugar, for example glucose which acts to stimulate insulin production in the body.

Eight, healthy, moderately trained, non-vegetarian men (age 22.3 ± 0.7 yr,

body mass 79.7 ± 2.5 kg, and body mass index 24.3 ± 0.9 kg/m^2) were used in the following study.

The study protocol utilised a blind crossover design where subjects acted as their own controls. Following an overnight fast, subjects reported to the laboratory on two occasions, separated by a 2 week "wash out" period to ensure similar basal muscle carnitine concentrations among experimental treatments. On arrival, subjects voided their bladder and were asked to rest in a supine position on a bed while a cannula was inserted retrogradely into a superficial vein on the dorsal surface of the non-dominant hand. This hand was kept in a hand-warming unit (air temperature 55°C) to arterialise the venous drainage of the hand and a saline drip was attached to keep the cannula patent.

After a basal blood sample was taken, subjects consumed 3.01 g (3 x 1.5 g L-carnitine L-tartare effervescent tablets) L-carnitine (Lonza Group, Basel, Switzerland) dissolved in 200 ml of water. After 1 hour and then 3 more times every 1.5 hours (h), subjects consumed a 500 ml drink over a 5 min period in a randomised order containing either sugar free orange drink (Control) or 94 g of simple sugars (CHO) (Original Lucozade, GlaxoSmithKline, Brentford, UK).

Subjects abstained from the consumption of meat, dairy produce, alcohol, and strenuous exercise 24 hours before each visit and for a 24 hour period after the consumption of the carnitine solution. It was essential that subjects had a minimal intake of carnitine in their diet during this period; therefore, food was supplied to the subjects as a ready-made meal, free from carnitine.

During each experimental visit, 5 ml of arterialised venous (a-v) blood were obtained every 20 min for 7 h after which subjects left the laboratory, returning for a final 24 hour blood sample. Two ml of this blood were collected into lithium heparin containers and, after centrifugation (14,000 rpm for 2 min), the plasma was removed and immediately frozen in liquid nitrogen. These samples were then stored at -80°C and analysed for free and total carnitine

concentrations at a later date. The remaining blood was allowed to clot, and, after centrifugation (3,000 rpm for 10 min), the serum was stored frozen at -20°C. Insulin concentration was measured in these samples at a later date with a radioammunoassay kit (Coat-a-Count Insulin, DPC, Ca, USA).

Urine was collected in 5 litre bottles, containing 5 ml of 10% thymol/isopropanol preservative, for 24 h following the consumption of the carnitine drink and returned to the laboratory the following morning where a final blood sample was taken. The 24 h volume was recorded and 5 ml aliquots were removed and stored at -20°C to be analysed for free and total carnitine concentrations at a later date.

The method used for the determination of carnitine is based on the carnitine acetyltransferase (CAT) catalysed reaction:

L-carnitine + [^{14}C]acetyl-CoA $\leftrightarrow$ [^{14}C]acetyl-L-carnitine + CoASH and measures the concentration of [^{14}C]acetyl-L-carnitine. The reaction is reversible, but the removal of CoASH via complex with N-ethylmalemide (NEM) ensures the reaction is driven quantitatively to the right and that all the L-carnitine is labelled. To separate labelled acetyl-L-carnitine from any remaining [^{14}C]acetyl-CoA Cederblad & Lindstedt (1972) introduced the use of anion-exchange resin. The negatively charged acetyl-CoA remains in the resin whereas the positive acetyl-L-carnitine is excluded for collection.

L-carnitine for use in the standards was purchased from Sigma Chemical Co., St. Louis, Mo. U.S.A., as was the unlabelled acetyl-coenzyme A (sodium salt, purity 90-95%), N-ethylmalemide, and the Dowex 1X 8 (200-400 mesh, Cl^- form). [^{14}C]acetyl-coenzyme A was obtained from Amersham, Buckinghamshire, UK (specific radioactivity 10 µCi). Carnitine acetyltransferase (5 mg/ml) was obtained from Roche Molecular Biochemicals, East Sussex, U.K. and scintillation liquid (Scintillator Plus) was purchased from Packard Biosciences, Groninger, The Netherlands.

All samples were analysed in duplicate. For plasma samples, 50 µl plasma were pipetted, by positive displacement, into a 3 ml glass test tube. After the addition of 1.2 ml chloroform/methanol ($CHCl_3$:CH_3OH, 3:2) the sample was vortexed, and then centrifuged at 4,500 rpm for 10 min. The supernatant was poured off to another glass tube while the pellet, after being broken up with a plastic rod, was ashed with a further 0.6 ml $CHCl_3$:CH_3OH, vortexed and centrifuged again (4,500 rpm, 10 min). This second supernatant was pooled with the first and the sample was dried by the evaporation of the $CHCl_3$:CH_3OH under N_2.

For total carnitine, all of the acyl-carnitine bonds were hydrolysed by the addition of 100 µl 0.1 M KOH to the test tube. The sample was then placed in a water bath at 50°C for 2 h. After incubation 20 µl of 0.5 M HCl were added to neutralised the sample.

For free carnitine, 120 µl H_2O (Millipor) were added to make the free and total solutions of equal volume.

For urine samples 10 µl urine were pipetted, by positive displacement, into a 3 ml glass test tube and diluted with 40 µl urine were pipetted, by positive displacement, into a 3 ml glass test tube and diluted with 40 µl H_2O (Millipor). The sample then underwent the same procedure as the plasma sample.

For the preparation of standards, 15, 30, 45, 60, 75, and 90 µl of 40 µmol/l L-carnitine standard solution were pipetted into 3.5 ml test tubes and made up to 120 µl volume by adding H_2O (Millipor). This produced 7 standards with L-carnitine concentrations of 0, 600, 1200, 1800, 2400, 3000, and 3600 pmol/l.

Radioenzymatic analysis of carnitine was carried out by adding twenty-five µl phosphate buffer (1 M, pH 6.5), 25 µl acetyl-CoA (300 µM), 10 µl NEM (40 mM), and 25 µl (^{14}C]acetyl-CoA (4 µM) to each tube. Carnitine acetyltransferase

(CAT) diluted 1:10 was then defrosted and 20 µl were added to each sample at 20 s intervals. After 30 min incubation at room temperature the contents of each tube were transferred to a column of Dowex 1X 8 contained in a Pasteur pipette at 20 s intervals using an automatic pipette (Microlab 1000, Hamilton, Bonaduz, Switzerland). The pipette aspirated each 240 µl sample with a 10 µl air gap, mixed with 250 µl water (Millipor) and then voided the solution into the top of column. The tube was then washed with 250 µl water (Millipor), which were then aspirated, mixed with 250 µl water (Millipor) and voided into the same column. The effluent was collected into 20 ml vials and mixed with 10 ml scintillation fluid. β-radioactivity of each of the vials was counted for 3 min.

A two-way ANOVA (time and treatment effects, SPSS version 10, USA) was performed to detect differences in plasma carnitine and serum insulin. A Student's paired *t*-test was used to locate differences in 24 h urinary carnitine content and area under plasma time curved between treatments. The total area under the plasma carnitine-time curve was calculated using KaleidaGraph (version 3.51, Synergy Software, USA). Statistical significance was declared at $P < 0.05$, and all the values are means ± SE.

The results will now be discussed with particular reference to the drawings.

Fig. 1 shows a plot of serum insulin concentrations following carnitine ingestion with Control CON (o) and carbohydrate CHO (•). Insulin concentration was significantly higher ($P<0.01$) following ingestion of four 500 ml drinks in the carbohydrate group (94 g simple sugars indicated by arrows A, B, C, D at t = 60, 150, 240 and 330) than in the control group (sugar free indicated by arrows t = 60, 150, 240 and 330). Values are ± SE expressed in mU/l (n = 8).

Fig. 2 shows a graph of urinary total carnitine (TC), free carnitine (FC) and acylcarnitine (AC) excretion in mg over a period of 24 hours following an oral

dose of 3.01 g of L-carnitine ingestion with control (CON) and carbohydrate (CHO). Mean urinary TC, FC and AC secretion was reduced when subjects consumed CHO compared to Control, and * indicates that excretion was significantly lower in the case of TC and AC ($P<0.05$). Values are means ± SE expressed in mg/24 hr (n = 8).

Fig. 3 shows a plot of plasma total carnitine concentration measured over 7 hours following an oral dose of 3.01 g L-carnitine with Control (□) and carbohydrate (■).

The arrows A, B, C and D indicate time of ingestion of drink. No significant differences ($P<0.05$) were seen between the two groups (Control and CHO), either at basal or at any point following ingestion. Values are means ± SE expressed in µmol/l (n = 8).

Fig. 4 shows a plot of the area under the plasma-time curves (AUC) for total carnitine (TC), free carnitine (FC) and acylcarnitine (AC) measured over a 7 hour period following an oral dose of 3.01 g L-carnitine with Control (CON) and carbohydrate (CHO). No significant differences were seen in TC and FC AUC's when comparing Control and carbohydrate, but AC was significantly lower ($P<0.05$) following CHO, resulting in a significant decrease in plasma carnitine concentration with CHO. Values are means ± SE expressed in mmol/l/min (n = 8).

The results show that L-carnitine supplementation together with CHO results in a smaller loss of urinary carnitine than that seen with Control. Total (TC), free (FC) and acyl (AC) carnitine were all excreted less with CHO, than in Control.

From the results it can be seen that insulin, released as a result of ingesting carbohydrate (CHO), stimulates L-carnitine retention. Insulin increases carnitine retention most probably by increasing sodium-potassium ATPase pump

activity and, thus, sodium dependent transport of carnitine into cells (particularly skeletal and cardiac muscle). Insulin may also enable more FC to be available to tissues by 1) inhibiting acylation of supplemented L-carnitine and/or 2) by stimulating carnitine retention by reabsorption by the kidney.

The present invention therefore has useful application in increasing carnitine retention in muscle tissue and thereby reducing the effects of depleted carnitine in muscle, including muscle fatigue and impaired muscular performance.

Various modifications may be made without departing from the spirit or scope of the present invention. For example other agents may be used which stimulate carnitine retention primarily by way of increasing carnitine transport into tissue, such as insulin or active derivatives thereof. Other agents may include, either as an alternative or as an addition, amino acid(s) and protein(s). Active derivatives, variants or analogues of carnitine may be used. The composition may be administered in any convenient form such as tablet, powder, pellet or the like and otherwise than by ingestion, such as injection.

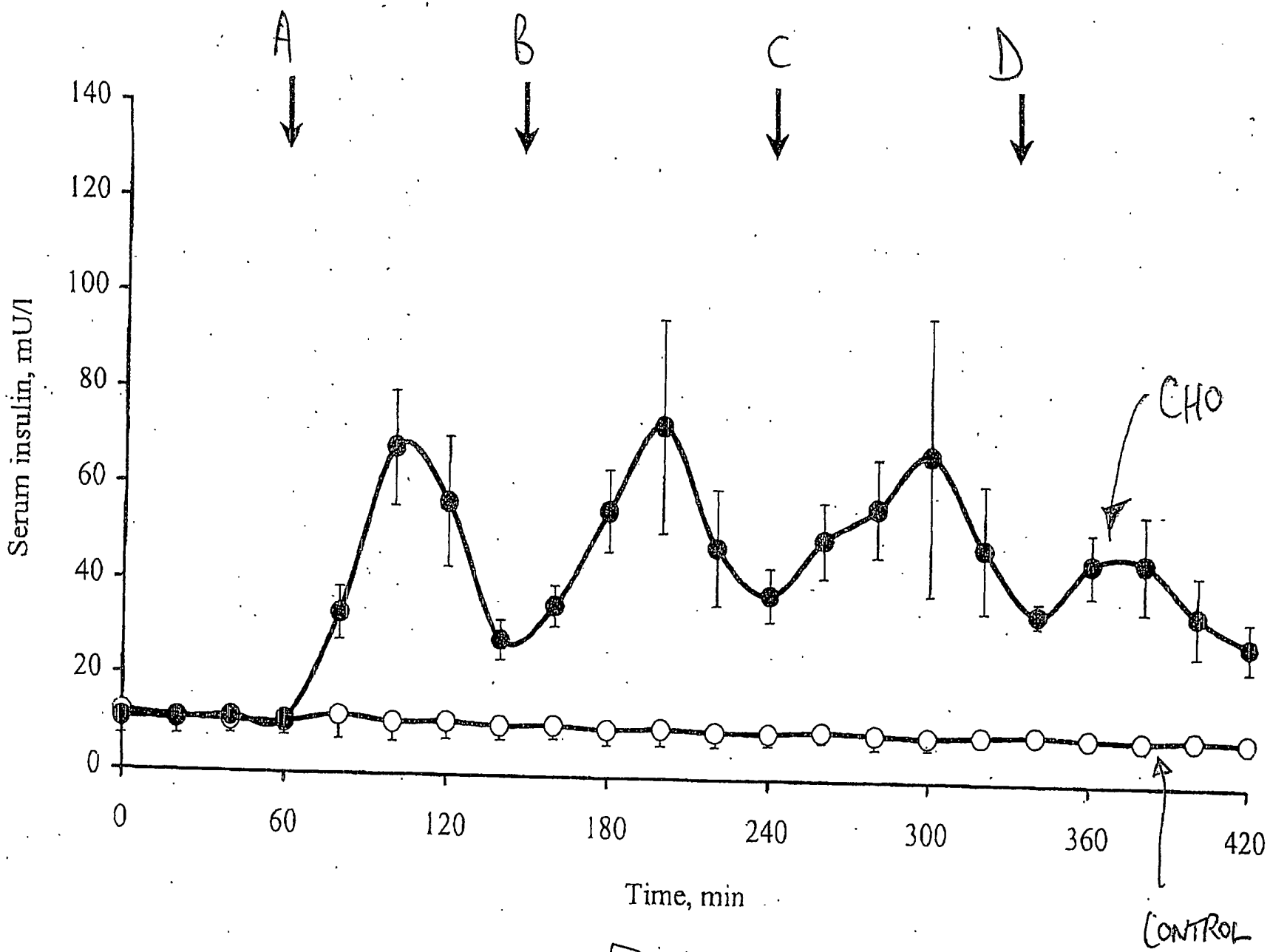
Between 10 and 150 times the amount by weight of agent such as carbohydrate may be administered to one unit of carnitine substance.

The invention can be used to increase carnitine retention in animal as well as human bodies, and in whole bodies, tissues or cells derived therefrom.

The invention also provides a kit comprising a carnitine substance and an agent such as a carbohydrate, as described above.

Whilst endeavouring in the foregoing specification to draw attention to those features of the invention believed to be of particular importance it should be understood that the Applicant claims protection in respect of any patentable feature or combination of features hereinbefore referred to and/or shown in the

drawings whether or not particular emphasis has been placed thereon.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B
C
D
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120
100
80
60
40
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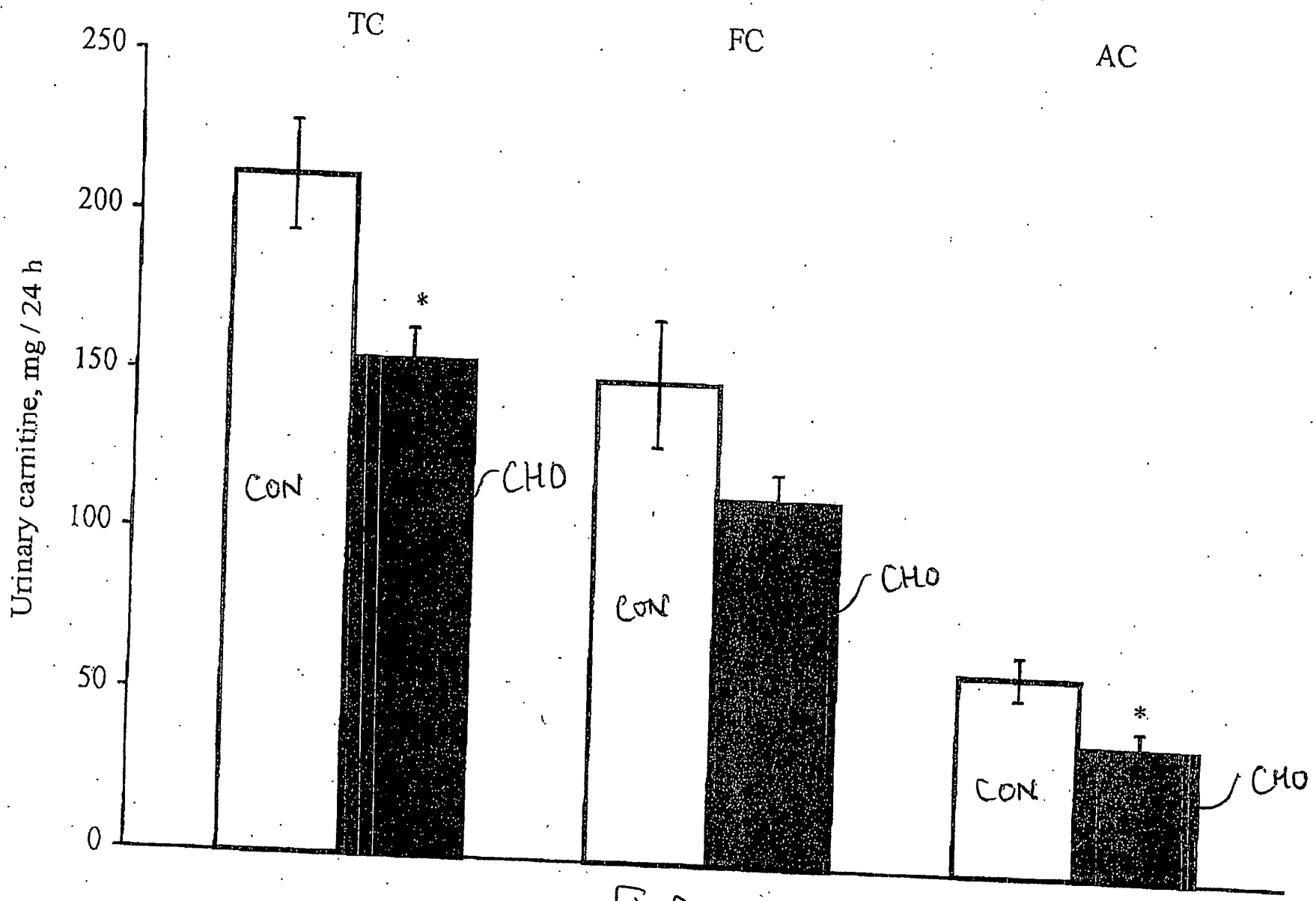
Serum insulin, mU/l
0
60
120
180
240
300
360
420
Time, min
CHO
CONTROL

Fig 1


TC
FC
AC
250
200
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100
50
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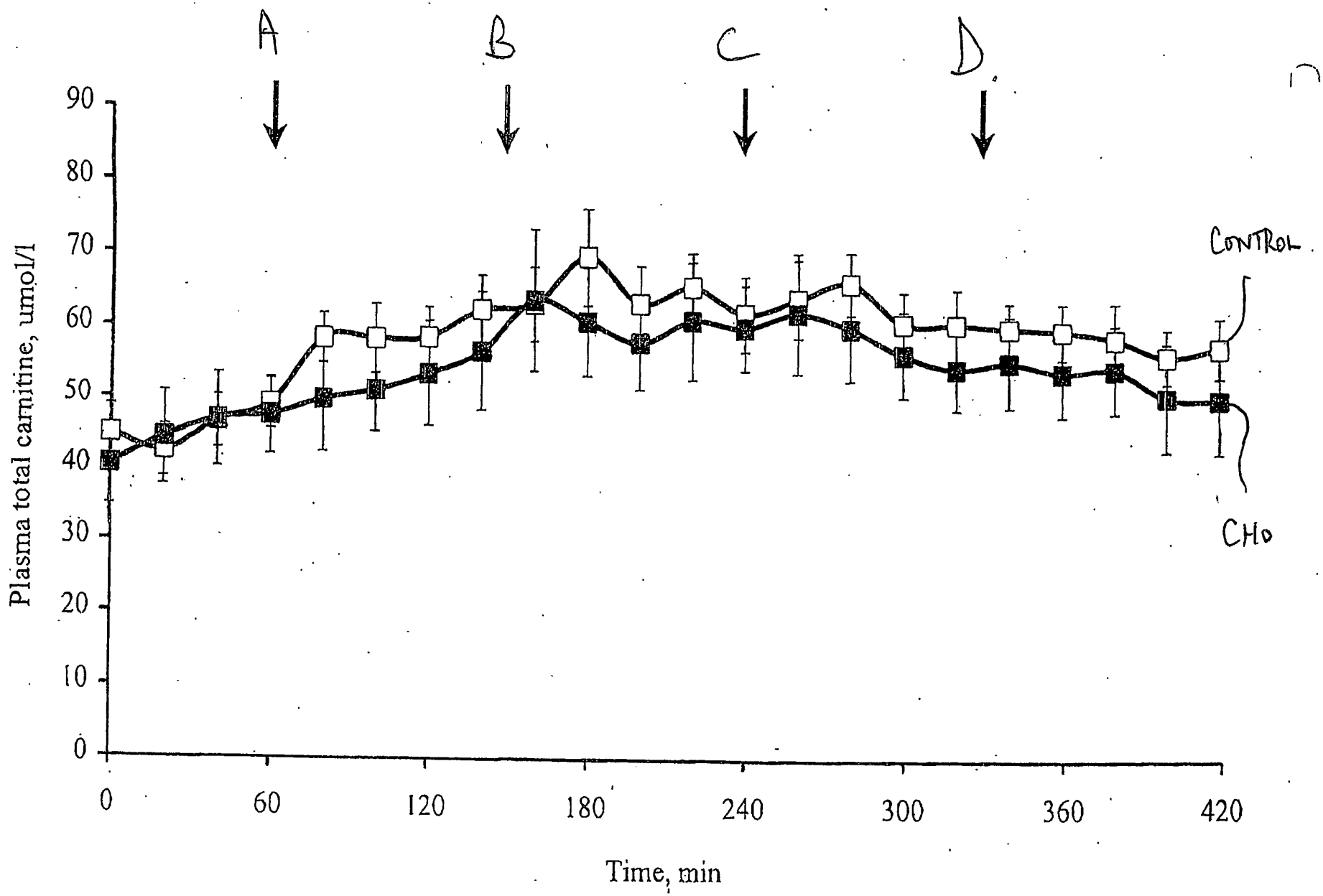
Urinary carnitine, mg / 24 h
CON
CHO
CON
CHO
CON
CHO
*
*

Fig 2


A
B
C
D
90
80
70
60
50
40
30
20
10
0
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CONTROL
CHO
0
60
120
180
240
300
360
420
Time, min

Fig 3

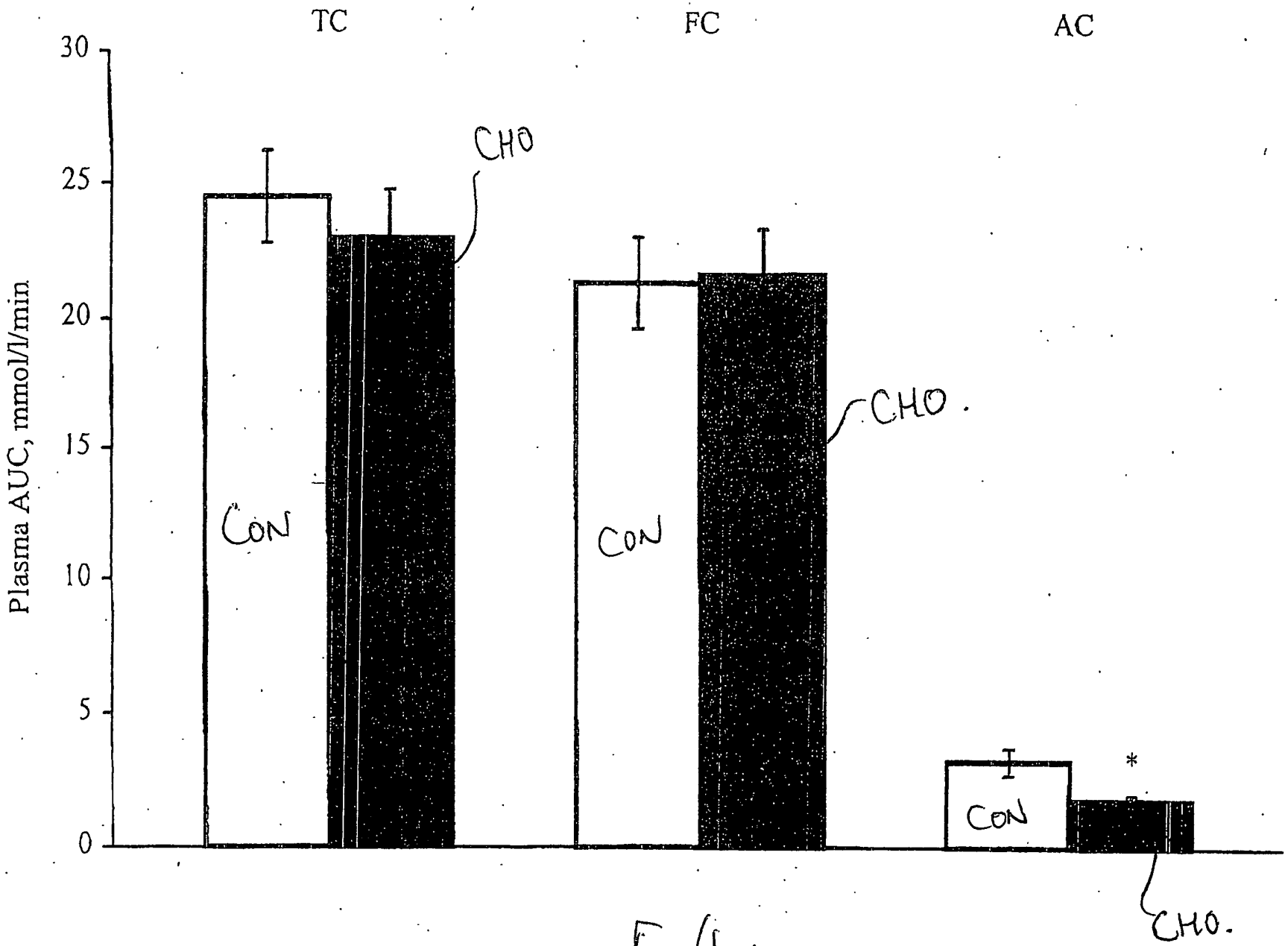
TC
FC
AC
30
25
20
15
10
5
0
Plasma AUC, mmol/l/min
CHO
CON
CON
CHO.
CON
*
CHO.

Fig 4.